PB80-194483

Chronic Effect of Copper on the Bluntnose Minnow, 'Pimephales notatus' (Rafinesque)

(U.S.) Environmental Research Lab.
Duluth, MN

12 Dec 78

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1. REPORT NO.	2.	3.
EPA-600/J-79-094	JOURNAL ARTICLE	PB80-194482

4. TITLE AND SUBTITLE	5. REPORT DATE
Chronic Effect of Copper on the Bluntnose Minnow, Pimephales notatus (Rafinesque)	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
W. B. Horning and T. W. Neiheisel	
9. PERFORMING ORGANIZATION NAME AND ADDRESS U.S. Environmental Protection Agency Environmental Research Laboratory-Duluth 6201 Congdon Boulevard Duluth, Minnesota 55804	10. PROGRAM ELEMENT NO. 1BA820 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Same as above	13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA-600/03

15. SUPPLEMENTARY NOTES

REFERENCE: Arch. Environ. Contam. Toxicol. 8, 545-552 (1979)

16. ABSTRACT

A laboratory chronic toxicity test in which bluntnose minnows were exposed to copper in laboratory dilution water with a hardness of 200 mg/L as $CaCO_3$ indicated that copper adversely affected fry survival, fry growth, and reproduction. The maximum acceptable toxicant concentration (MATC) for total copper based on reproductive impairment at 18.0 µg/L was between 4.3 (control concentration) and 18.0 µg/L.

The 96-hr LC50 values from three acute toxicity tests ranged from 0.22 to 0.27 mg/L total copper with a mean value of 0.23 mg/L. The application factor (MATC/96-hr LC50) for bluntnose minnows and total copper was estimated to lie between 0.02 and 0.08.

Bluntnose minnows held in control water for nine months ceased to spawn when they were exposed to 119.4 µg/L total copper. Fish exposed to 119.4 µg/L total copper for the same nine-month period began to spawn 60 days after being transferred to control water.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS		b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Bioassay	Freshwater	Acute toxicity	06/F
Bluntnose minnow	Fish	Chronic toxicity	
Survival	Aquatic life	Copper	
Growth			
Reproduction			
Metals			

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NATIONAL TECHNICAL
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SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	
	20. SECURITY CLASS (This page)	22. PRICE
	UNCLASSIFIED	

EPA-600/J-79-094
JOURNAL ARTICLE

Arch. Environm. Contam. Toxicol. 8, 545–552 (1979)

Archives of Environmental Contamination and Toxicology

Chronic Effect of Copper on the Bluntnose Minnow, *Pimephales notatus* (Rafinesque)

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Abstract. A laboratory chronic toxicity test in which bluntnose minnows were exposed to copper in laboratory dilution water with a hardness of 200 mg/L as $CaCO_3$ indicated that copper adversely affected fry survival, fry growth, and reproduction. The maximum acceptable toxicant concentration (MATC) for total copper based on reproductive impairment at 18.0 μg/L was between 4.3 (control concentration) and 18.0 μg/L.

The 96-hr LC50 values from three acute toxicity tests ranged from 0.22 to 0.27 mg/L total copper with a mean value of 0.23 mg/L. The application factor (MATC/96-hr LC50) for bluntnose minnows and total copper was estimated to lie between 0.02 and 0.08.

Bluntnose minnows held in control water for nine months ceased to spawn when they were exposed to 119.4 μg/L total copper. Fish exposed to 119.4 μg/L total copper for the same nine-month period began to spawn 60 days after being transferred to control water.

The purpose of this chronic toxicity test was to determine the effects of continuous copper exposure on survival, growth, and reproduction of the bluntnose minnow, *Pimephales notatus* (Rafinesque). Another objective of the study was to determine if the effect of copper on bluntnose minnow spawning that was observed by Geckler *et al.* (1976) during intermittent exposure in field tests could be repeated under standard laboratory conditions. Geckler and his co-workers found that bluntnose minnows did not spawn while being exposed to 240 μg/L copper during a streamside chronic toxicity test in which a natural surface water was used for dilution. After the addition of copper to the test system had ceased for 30 days, the fish began to spawn. When copper was again added, spawning ceased.

The bluntnose minnow was chosen for this study, because it is a ubiquitous stream species, because it has been used in only a few laboratory studies, and because we believed that the species might have promise as another bioassay test animal.

0090–4341/79/0008–0545 $01.60
1979 Springer-Verlag New York Inc.

Methods and Experiment

Exposure System

Since the bluntnose minnow and the fathead minnow are closely related and behave similarly, the methodology and experimental design essentially followed the procedures described by Mount and Stephan (1967) and Mount (1968). The toxicant delivery system was a proportional diluter (Mount and Brungs 1967) modified to deliver five toxicant concentrations (nominally 120, 72, 43, 26, and 16 μg/L copper) and control water to duplicate sets (A and B) of glass chambers, and to provide an additional duplicate set of control and high concentration chambers. Each 60- × 30- × 30-cm test chamber contained approximately 30 L of water. The flow of water to each chamber was approximately 10 volumes per day. A glass fry-rearing chamber (30 × 15 × 15 cm) which contained 10 L of water was placed in front of each test chamber. Approximately 100 L of water per day flowed from each adult fish chamber into each fry chamber.

Six 7.6-cm half-tiles (made of cement and asbestos) were placed in each adult test chamber for spawning substrates. Eggs were hatched in cups that were suspended in the adult test chambers from a rocker arm that oscillated two times per minute (Mount 1968).

Physical and Chemical Conditions

The experimental diluent water was a mixture of spring water obtained from the Newton Fish Farm (a Hatchery of the Ohio Division of Wildlife, Newtown, Ohio) and carbon-filtered, demineralized tap water from Cincinnati, Ohio. The water was maintained at a total hardness of 200 mg/L as $CaCO_3$ (EDTA Method) and was stored in a 400,000-L concrete reservoir covered with translucent fiber glass panels. Retention time in this reservoir was approximately 30 to 40 days. During the study, dissolved oxygen concentrations were 5.9 mg/L or greater; pH ranged from 7.88 to 8.31; alkalinity ranged from 150 to 186 mg/L and total hardness ranged from 172 to 230 mg/L.

Photoperiod was controlled to simulate the natural daylight cycle for southern Ohio. Four fluorescent light fixtures with two 48-inch, 40-w lamps were suspended one m above the test system. Each fixture contained a General Electric "Gro-lux" lamp and a Sylvania "Optima" lamp.[1] Temperature was recorded continuously with a seven-day indicating and recording thermometer.

The stock solution of copper used in the toxicant delivery system was made by dissolving reagent-grade copper sulfate ($CuSO_4 \cdot 5H_2O$) in demineralized water. The chemical metering system was checked daily to assure that the proper amount of stock toxicant solution was being delivered. The system delivered the desired amount 97.4% of the time. Samples of water (100 ml) were removed daily for analysis from one set of duplicate test chambers and were composited for seven days. Each set of duplicate chambers was sampled on alternate weeks. The composite samples were analyzed for total copper concentration by atomic absorption spectrophotometry as described by Brungs *et al.* (1973) (Table 1). There was no appreciable difference in total copper concentrations between any of the duplicate chambers.

The standard addition method was used on one composite sample each week to determine analytical accuracy. Recovery of copper from 62 spiked duplicate samples averaged 108% ± 14.

Samples collected weekly from one set of duplicate test chambers were analyzed for dissolved oxygen, pH, alkalinity, and total hardness. Each set of chambers was sampled on alternate weeks. The chemical analyses were conducted according to methods described by the American Public Health Association (1971).

Biological System

Bluntnose minnow eggs were collected from Shayler Run Creek (near Newtown, Ohio) and were

[1] Mention of trade names or commercial products does not constitute endorsement or recommendation for use

Table 1. Total copper analyses

Measured concentration (μg/L)	Duplicate chambers	Measured[a] concentration (μg/L)	Mean measured concentration (μg/L)
Control	A	4.1 ± 1.7	4.3
	B	4.6 ± 1.7	
16	A	18.2 ± 2.6	18.0
	B	17.9 ± 2.1	
26	A	29.3 ± 3.8	29.9
	B	30.6 ± 3.2	
43	A	43.8 ± 5.9	44.1
	B	44.5 ± 4.4	
72	A	71.2 ± 7.6	71.8
	B	72.6 ± 5.3	
120	A	118.4 ± 9.0	119.4
	B	120.6 ± 9.0	

[a] Mean values based on 29 to 34 weekly composite samples

hatched at the Newtown Fish Toxicology Station laboratory. On September 9, 1973, twenty-two 6-week-old fry (15-16 mm long) were randomly assigned to each of 16 test chambers. The fish were treated for three consecutive days with neomycin sulfate (15 mg/L), tetracycline (15 mg/L), and Dexon (40 mg/L); the addition of copper started the day after the last prophylactic disease treatment.

During the last week in April 1974, the fish were separated according to sex and thinned to a sex ratio of five males and ten females per chamber. However, only 13 fish had survived in one control chamber and 14 had survived in the duplicate 18 μg/L chambers.

Tail rot occurred sporadically throughout the test. Dead or badly infected animals were removed, and the remainder were treated in the manner described earlier.

During the spawning season, the tiles were examined daily, and all eggs were removed and counted. Fifty eggs were placed in an egg cup, and the rest of the eggs were discarded. When a spawn of less than 50 eggs occurred, all of the eggs were placed in an egg cup. The eggs were incubated in the same copper concentration in which they were spawned. Additionally, eggs produced in the control chambers were transferred to 29.9, 44.1, 71.8, and 119.4 μg/L copper concentrations for hatchability determination. During the first four days, dead eggs were removed, incubating eggs were cleaned, and the number of viable eggs recorded. Hatching normally began in four days, and generally all were hatched in seven days. The number of live larvae on the seventh day determined the hatch percentage. Larvae from eggs hatched in the control chambers were transferred to 29.9, 44.1, 71.8, and 119.4 μg/L copper concentrations, to determine effect on growth.

After approximately two months of spawning activity, the fish in the extra control tanks were exposed to 119.4 μg/L total copper for the remainder of the test. At the same time the fish that were being exposed in the extra 119.4 μg/L total copper chambers were changed to control water for the remainder of the test.

When the study was terminated, the testes of the mature male fish were macroscopically and microscopically examined. In addition, ovaries of the mature females were macroscopically examined, and the gross condition of the eggs was determined.

During April 1974, bluntnose minnows were collected from Shayler Run Creek for a 96-hr acute flow-through toxicity test in which a proportional diluter and experimental laboratory water was used. The test temperature was maintained at 25°C. Ten fish were used in duplicate exposure

chambers; thus, 20 fish were used for each copper concentration. Water samples were taken at the end of each test for copper analysis. Two additional 96-hr flow-through tests were conducted during April 1975. Fish for these tests were from eggs that had been spawned and hatched in the control chambers during the chronic study. Results from the acute tests are based on measured values of total copper concentration.

A computer program combining one-way analysis of variance (ANOVA) ($P = 0.05$) and Duncan's multiple range test ($P = 0.05$) (Duncan 1955) was used to evaluate the survival, growth, egg-production, and hatchability data. Untransformed data were used in the ANOVA and the Duncan's multiple range test. Probit analysis (Finney 1971) was used to calculate the LC50 values.

Results

Acute Toxicity

The 96-hr flow-through LC50 measured total copper value for bluntnose minnows collected from Shayler Run in April 1974 was 0.21 mg/L. Measured total copper LC50 values for the bluntnose minnows hatched and raised in the experimental water at 200 mg/L hardness (as $CaCO_3$) were 0.22 and 0.27 mg/L. Experimental laboratory water was used for all three tests. The mean LC50 value for the three tests was 0.23 mg/L

Chronic Toxicity

After 30 days, the parental fish exposed to 119.4 μg/L total copper were significantly smaller (mean 25.5 mm, $P = 0.05$) than those exposed to 44.1, 29.9, and 18.0 μg/L total copper and to control water (30.2-31.6 mm). The fish exposed to 71.8 μg/L total copper were also significantly smaller (27.9 mm, $P = 0.05$) than those exposed to 18.0 μg/L and the controls. Bluntnose minnows exposed to 119.4 μg/L total copper for 60 days were significantly smaller (35.9 mm, $P = 0.05$) than those exposed to all of the other copper concentrations (40.6-46.6 mm). The fish exposed to 18.0 μg/L total copper were somewhat larger than all the others, including control animals.

Analysis of variance of the growth data indicated that copper significantly ($P = 0.05$) affected the growth of second-generation bluntnose minnows after 30 and 60 days' exposure. These fish had been hatched in control chambers and then transferred to 29.9, 44.1, 71.8, and 119.4 μg/L total copper concentrations. Duncan's multiple range test indicated that after 30 days the fish exposed to 119.4 μg/L total copper were significantly smaller (11.2 mm, $P = 0.05$) than those in all other concentrations (13.0-15.6 mm). Also, the fish exposed to 71.8 μg/L total copper (13.0 mm) were significantly smaller than those exposed to 29.9 μg/L (14.6 mm). However, after 60 days' exposure to copper only the growth of fish exposed to 119.4 μg/L was significantly affected.

Survival of parental bluntnose minnows was not affected by any copper concentrations during the initial 60 days of exposure. However, survival of fish exposed to 119.4 μg/L total copper was lower at the end of the test (87%) than survival of fish exposed to the other copper concentrations (93-100%). Survival of second-generation bluntnose minnows was affected by copper more quickly than survival of parental fish. Duncan's multiple range test indicated that survival of fish exposed to 119.4 μg/L copper was significantly ($P = 0.05$) less

(44.7% and 38.2%) after 30 and 60 days' exposure than for fish exposed to the other copper concentrations (81.5-89.0% survival after 60 days).

Spawning began in the control chambers during mid-May 1974 and continued at a fairly uniform rate in one test chamber (A) through mid-December 1974. Spawning was sporadic in the other (B) control chamber during June 1974 and continued at a fairly uniform rate during July and August 1974. Spawning began in the 18.0 μg/L exposure chambers during the first week in June 1974, and continued in chamber A at a fairly uniform rate during July and August 1974, but was sporadic in chamber B. Spawning was sporadic in both chambers and tapered off during September and October 1974 and was completed by mid-November 1974. Spawning began in one 29.9 μg/L exposure chamber (B) during the first week in June 1974 and continued sporadically through the end of October 1974. Only three spawnings occurred in the other chamber (A) during the test. Spawning did not occur in the high copper concentrations.

The mean water temperature was 24.7°C, with a range from 16° to 27°C until June 20, 1974. Because spawning was sporadic in control chamber B and the 18.0 μg/L exposure chamber B and was totally lacking in the 29.9 μg/L exposure chamber A during the first three weeks in June 1974, we decided to decrease the temperature to 23°C, since the bluntnose minnow may have a lower optimum spawning temperature than the fathead minnow, which spawns well in the laboratory at 25°C. The mean water temperature for the remainder of the test was 22.7°C, with a range from 21° to 25°C.

Copper significantly reduced the number of eggs produced per female. Duncan's multiple range test verified that the mean number of eggs produced per female in the control chambers (315) was significantly greater ($P = 0.05$) than the number of eggs produced per female at all of the test concentrations (Table 2).

Table 2. Spawning and egg production of the bluntnose minnow at various copper concentrations

Mean total copper concentration (μg/L)	Number of females	Number of spawnings	Total eggs produced	Number of eggs/ female
4.3	10	69	3688	369 (315)[a]
(Control)	8	37	1985	248
18.0	8	21	708	89 (62)
	7	12	229	33
29.9	10	3	15	2 (20)
	8	16	343	43
44.1	5	3	19	4 (4)
	7	2	34	5
71.8	10	0	0	0 (0)
	5	1	3	1
119.4	3	1	4	1 (0)
	2	0	0	0

[a] Mean value.

Spawning of the bluntnose minnows held in control water for about nine months ceased after exposure to 119.4 μg/L total copper was begun. The mean number of eggs produced per female was 53 during the two months these fish were spawning before the addition of copper. Fish that had been exposed to 119.4 μg/L total copper for about nine months began to spawn 60 days after being transferred to control water. The mean number of eggs produced per female by these fish was 97.

Egg hatchability was determined only for the control and the 18.0 μg/L copper concentrations, since not enough eggs were produced in the other concentrations to determine hatchability. Hatchability was not significantly different between the two groups of fish. Copper did not appear to affect the hatchability of eggs produced in the control chambers and incubated in 29.9, 44.1, 71.8, and 119.4 μg/L total copper concentrations.

Examination of the gonads at the end of the study revealed that male bluntnose minnows exposed to 18.0 and 29.9 μg/L total copper and those exposed to control water (4.3 μg/L total copper) were sexually mature. Their testes were filled with motile sperm. Several of the males exposed to 44.1 μg/L total copper were immature, but the testes in the mature individuals were full of motile sperm. Most of the males exposed to 71.8 μg/L total copper were immature, and the number of motile sperm in the testes was markedly reduced. All males exposed to 119.4 μg/L total copper were sexually immature with poorly developed testes. Eggs were present in the ovaries of female bluntnose minnows exposed to all copper concentrations, but they appeared to be either poorly developed or were being resorbed. In addition, most of the females exposed to 71.8 and 119.4 μg/L total copper were sexually immature.

Sexually mature male fish, transferred from control water (4.8 μg/L total copper) to an exposure of 119.4 μg/L total copper after spawning had begun, regressed to a condition of poorly developed testes and no motile sperm. At the end of the test, several fish were immature and could not be identified as male or female. Females that were transferred from control water to 119.4 μg/L total copper contained eggs that appeared to be resorbing, and no further spawning occurred with these fish. As indicated previously, fish exposed to 119.4 μg/L total copper for nine months began to spawn 60 days after being transferred to control water (4.3 μg/L total copper). The male fish were sexually mature at the end of the study and their testes contained many motile sperm. The females were also sexually mature, but some egg resorption was occurring. Thus, exposure to 119.4 μg/L total copper before the onset of spawning did not adversely affect the reproductive capability of the bluntnose minnows in this study. However, exposure to 119.4 μg/L total copper after spawning was initiated did affect reproduction, since spawning was completely stopped.

Discussion

Copper affects fry survival, fry growth, and reproduction of bluntnose minnows, but because of poor spawning in the test copper concentrations, no data could be collected on the hatchability of eggs and growth of fry from adult fish that were exposed to copper. Reproductive impairment was demonstrated in

the lowest copper concentrations. These results agree with studies by Mount (1968) and Mount and Stephan (1969), which showed that copper primarily affects reproductive success of fathead minnows.

Results from this study contrast sharply with those from chronic toxicity tests conducted by Geckler *et al.* (1976), in which surface water from Shayler Run was used. They reported that bluntnose minnow egg production was not reduced by total copper concentrations of 107 and 112 μg/L, whereas in the present study bluntnose minnow egg production was significantly ($P = 0.05$) reduced by 18.0 μg/L total copper. This discrepancy may be explained by the differences in water supplies. Phosphates and chelating agents were present in the Shayler Run water, but not in the experimental water.

Brungs *et al.* (1976) reported that the Maximum Acceptable Toxicant Concentration (MATC) for fathead minnows and copper was approximately 66 μg/L, when Shayler Run water was used for dilution. Geckler *et al.* (1976) also reported that in two additional chronic toxicity tests, in which Shayler Run water was used, fathead minnow egg production was significantly reduced ($P = 0.05$) by total copper concentrations of 117 and 112 μg/L. The MATC in these two tests was between 65 and 117 and between 59 and 112, respectively; whereas the MATC for bluntnose minnows in Geckler's test was greater than 112 μg/L total copper. This indicated that bluntnose minnows were more resistant than fathead minnows to the chronic effects of copper when a natural surface water was used for dilution. In contrast, a study by Mount (1968), in which a laboratory water supply was used, indicated that fathead minnows were somewhat more resistant to the chronic effects of copper than the bluntnose minnows used in the present study. Mount found the MATC for copper and fathead minnows to be between 15 and 33 μg/L total copper. The present laboratory study indicates that the MATC for copper and bluntnose minnows is between 4.3 (mean control total copper concentration) and 18.0 μg/L.

In flow-through acute toxicity tests conducted in experimental laboratory water (hardness 200 mg/L as $CaCO_3$), bluntnose minnows were somewhat more sensitive to copper than fathead minnows. Geckler *et al.* (1976) reported that the 96-hr LC50 was 0.34 mg/L total copper for bluntnose minnows and was 0.44 and 0.49 mg/L for two tests with fathead minnows. He also reported that the sensitivity of bluntnose minnows and fathead minnows to copper was essentially the same when the acute toxicity tests were conducted with Shayler Run water. The 96-hr LC50 values ranged from 2.4 to 11.0 mg/L and 4.1 to 13.0 mg/L copper for the bluntnose minnows and fathead minnows, respectively. The 96-hr LC50 values during the present study were somewhat lower than those reported by Geckler *et al.* (1976). Our values ranged from 0.22 to 0.27 mg/L measured total copper with a mean value of 0.23 mg/L. Geckler *et al.*, using a static acute toxicity test, reported values from 0.26 to 0.29 mg/L total copper, with a mean of 0.27 mg/L.

The application factor described by Mount and Stephan (1967) is the ratio of the MATC for the toxicant of concern to the 96-hr LC50. The MATC for the bluntnose minnows under the described test condition is between 4.3 and 18.0 μg/L total copper, and the mean LC50 from three flow-through acute toxicity tests is 0.23 mg/L total copper. If we use these values, the application factor for the bluntnose minnow and copper is between 0.02 and

0.08. Application factors of 0.03-0.07 and 0.13-0.22 for fathead minnows and copper in hard and soft water, respectively, were determined by Mount (1968) and Mount and Stephan (1969). McKim and Benoit (1971) determined the application factor for brook trout and copper to be between 0.10 and 0.17 in soft Lake Superior water. The agreement between these application factors is good when inherent biological variability and other sources of error are considered.

Based on this study and the study done on Shayler Run (Geckler *et al.* 1976), it appears that the bluntnose minnow is an acceptable species for use in chronic studies for evaluation of toxicity. These studies indicate that the bluntnose minnow can be easily cultured and raised under laboratory conditions through a complete life cycle. This is an extremely important factor in determining the suitability of an organism for chronic toxicity testing.

Acknowledgments. The authors gratefully acknowledge the efforts of Marion Gast (deceased), who actually performed the study. The authors also wish to thank James Dryer, who performed the statistical analysis of the data.

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Manuscript received December 12, 1978; accepted January 25, 1979.